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NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

The present invention relates to a method of detecting specific target DNA sequences, and in particular to the products of amplification reactions, as well as to reagents and apparatus used in that method.

Many methods are known in order to detect the presence of particular target DNA sequences in a sample. A substantial proportion of these methods require that the DNA is denatured to single stranded form and then this sequence is hybridised or otherwise allowed to bind to a labelled sequence specific probe.

The target sequences are frequently subjected to amplification reactions, for example the polymerase chain reaction or the ligase chain reaction, in order to increase the amount of the target sequence to detectable levels.

Other methods of detecting sequences include the use of intercalating dyes which are incorporated into the sequences during the amplification reaction. However such methods are relatively non specific as the dyes will intercalate with any amplification product, even if they are the result of non-specific amplification products.

Other assays such as the TAQMAN™ assay utilise complex probes which include reporter and quencher moieties during the course of the amplification process. These probes hybridise to single stranded target sequences during the amplification reaction and are then digested by the enzymes carrying out the reaction. The relationship between quencher and reporter molecule of the probe produces a signal which can be monitored. The probes used in this case however, are complex and expensive.

It is known that peptide nucleic acids will strand invade DNA at purine rich sites to form triplex structures (P.E. Nielson et al., Science, 1991, 254, p1497-1506, Turney D.Y. et al,. Proc. Natl. Acad. Sci. USA , 1993, 90, 1667-1670). The mechanism by which this is effected is illustrated diagrammatically hereinafter in Figure 1.

The applicants have found that this phenomenon can be used in detection of target DNA sequences.

10 A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

The method enabling the direct detection of target sequences, for example amplification products without the usual denaturation step required for duplex formation with a nucleic acid probe.

The expression "purine rich region" means that the sequence is suitable for strand invasion by a peptide nucleic acid (PNA). Such regions suitably contain at least four consecutive purine residues.

The reaction in step (a) above is suitably effected in the presence of a buffer, and preferably a low salt buffer for example containing 50mM or less of salt as this favours triplex formation as compared to DNA:DNA duplexes. Furthermore, the pH of the buffer used will depend on the precise nature of the PNA

employed. If C's are used in the PNA strand to strand invade G's on duplex DNA, careful consideration has to be given to the pH of the buffer as the C involved in forming the Hoogsteen base-pair needs to be protonated, requiring a buffer of low pH, for example of less than 4.5.

The peptide nucleic acid used in the method of the invention may be single stranded or it may be bis-PNA. Preferably, the peptide nucleic acid used in the method is a bis-PNA as this results is a faster strand invasion process and a more stable triplex product.

Bis-PNA will comprise of two anti-parallel strands joined by a hydrophilic linker. One strand will be designed for Watson-Crick recognition of DNA within the target sequence, and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex. Such acids will be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

Peptide nucleic acids used will suitably contain a sequence of poly-T's or poly-C's.

The target nucleic acid is first subject to an amplification reaction such as the polymerase chain reaction (PCR) or ligase chain reaction (LCR), preferably PCR. The product may be exposed to the peptide nucleic acid during or after the amplification reaction, but is preferably exposed to the peptide nucleic acid after completion of the amplification reaction.

Where the target nucleotide sequence contains or is selected such that it contains a purine rich region, the method can be carried out directly. Where such regions do not exist in the target sequence, they may be introduced during the amplification

reaction. In this case, the amplification will be effected using one or more primers which comprise a plurality of pyrimidines, suitably at the 5' end thereof. This region will chain extend during the extension phase of the amplification (as illustrated in Figure 2 hereinafter). The 3'-end of both amplified strands of the amplification obtained using these primers should now contain the purine rich sites. Indeed, PCR products, that were tagged in this manner, have been cloned and sequenced and were found to have the poly-purine stretches incorporated at their 3'end. This ensures that a suitable PNA binding purine rich region is contained within the amplification product.

Primers of this sort form a further aspect of the invention.

The triplex formed may be detected using various methods in step (b). For example, gel retardation methods may be used. When the product is subjected to gel electrophoresis, for instance on a non-denaturing polyacrylamide gel, and then stained using conventional reagents such as ethidium bromide, the presence of a retarded triplex fraction can be observed.

This method however is relatively slow. Furthermore, comparison with a similar sequence which is not in the form of a triplex is required as a standard.

Preferably therefore, the detection is effected using a capture assay. The capture agent in this case is suitably the PNA sequence which is immobilised on a support. The sample is then contacted with the support whereupon any target sequence present will become associated with the PNA on the surface. It can then be detected using any of the known techniques.

In a particularly preferred embodiment, the support is a waveguide of a detection device which operates using evanescent wave detection. An example of such a device is a surface plasmon resonance detector. This allows the direct and rapid detection of target nucleotide sequence within a sample.

Thus a product of the amplification reaction is simply allowed to flow over the waveguide of such a detector and the presence of an amplicon can be detected in something approaching "real time".

In a further aspect, the invention provides a kit for use in the method of the invention. These kits suitably comprise a PNA designed to form a triplex with a target DNA. Optionally also, it may contain primers which can be used in the amplification of the target DNA, in particular primers which are 5'-tagged with pyrimidines.

The kit may also comprise a waveguide of a evanescent wave detector and particularly a surface plasmon resonance detector having supported thereon, the peptide nucleic acid which specifically binds a target DNA sequence.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 illustrates diagrammatically PNA:DNA triplex formation;

Figure 2 illustrates diagrammatically the incorporation of purine rich regions into an amplification product, using 5'-tagging of primers with polyramidine sequences; and

Figure 3 illustrates triplex formation on the surface of a surface plasmon resonance detector.

Example 1

Triplex Formation

The ability of PNA to form triplex structures with PCR products has been demonstrated using gel retardation studies. Two PCR products were chosen for study. One has a sequence capable of forming triplexes with a PNA probe i.e. contains poly-A sites.

PCR82

5'

ATAAATACAACCAACAAAATAAATAGTCATAAAATTGTATACATTAGCAATGCATACC ACAAAGTTCTAAGTACTAAAATAT 3' (SEQ ID NO 1)

The other does not contain poly-A sites and acts as a negative control.

PCR 175

5'

GCGAAACGGAACATAGCCCAAACCAAGAGGCTTGCCTCTTGGGGTTGTAGGACATTCT ATACGGAGTTACAAAGGAAGCAGGTAGACGAAGCGACCTGGAAAGGTCCGTCGTAGAGGGTAAC AACCCCGTAGTCGAAACTTCGTTCTCTCTTGAATGTATCCTGAGTACGGCGGAACACGTGAAA 3' (SEQ ID NO 2)

Two types of PNA probe were used, one was a linear sequence and contains a sequence of poly-T's

PNA057

N TTTTCCTTCCCTTTT C (SEQ ID NO 3)

The other, a bis-PNA of the same linear sequence but composed of two anti-parallel strands joined by a hydrophilic linker. One

strand was designed for Watson-Crick recognition of DNA and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex and should be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

PNA058

N TTTTCCCTTCCTTTT LLL TTTTCCTTCCCTTTT C (SEQ ID NO 4)

Each PCR product (5 µg/ml) was incubated with each PNA probe (10 µg/ml), at 37°C in 0.5 X TE buffer (1 mM Tris.HCl, 0.1 mM EDTA, 5 mM NaCl, pH 8.0) for varying time intervals before the reaction was terminated by adding 150 mM HBS, pH 7.4 on ice. Samples were run on a non-denaturing 12% polyacrylamide gel. The electrophoretic mobility of the triplex PNA_2DNA was compared to the duplex DNA of the relevant PCR product and visualised by EtBr staining. Triplex structures were observed suggesting that PNA can directly detect double-stranded PCR products.

The results of the gel retardation studies showed that single-stranded PNA did not strand invade the PCR products within the first 60 minutes. (This is backed up in the literature where it has been demonstrated that the association of a bis-PNA with a single strand of homopurine DNA gives a complex that is significantly more stable than the one formed with two single PNA strands due to a more favourable entropy of reaction.)

Bis-PNA, however, formed a triplex within the first 10 minutes of reaction.

Example 2

Detection of triplexes on a surface plasmon resonance (SPR) surface.

Biotin labelled bis-PNA (50 µg/ml) was linked to a dextran surface (Biacore, SAchip) via a streptavidin-biotin interaction. A sample of both PCR products (10 µg/ml), in water, was flowed

over this sensor surface and were detected by a change in refractive index. The SPR system could differentiate between purine-rich and non-purine rich PCR products in near real time (See Figure 3).

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